β-Nerve Growth Factor in Developing Mouse Cerebral Cortical Synaptosomes: Measurement by Competitive Radioimmunoassay and Bioassay

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ABSTRACT. The existence of β-nerve growth factor (βNGF) in mouse cerebral cortical synaptosomal extracts was assessed using a newly developed radioimmunoasay and by bioassay. Displacement curves of labeled βNGF in the radioimmunoassay by synaptosomal extracts were parallel to the βNGF standard curves. In contrast, both mitochondrial and nonsynaptosomal extracts produced displacement curves in the βNGF radioimmunoassay which were nonparallel to standard βNGF. Intraventricular injections of purified βNGF in amounts 5 and 100 ng/mouse (six to 120 times that of whole brain βNGF concentrations) did not alter the endogenous synaptosomal βNGF concentrations. Synaptosomal extracts contained βNGF in relatively higher concentrations than the extracts of whole brain or cerebral cortex. Synaptosomal βNGF levels undergo a developmental change. The βNGF concentrations were highest in synaptosomal extracts of 12-day-old mouse cerebral cortex (the youngest age studied), and levels decreased with increasing age. Synaptosomal extracts of 12-day-old mouse cerebral cortex produced neurite outgrowth in the pheochromocytoma cell line 12 bioassay system and this response was completely inhibited by βNGF antibodies. In the pheochromocytoma cell line 12 cell bioassay, synaptosomal extracts also caused cell-cell aggregation which was unaffected by βNGF antibodies. These results provide evidence for the existence of βNGF in relatively high concentration in nerve terminals of mouse cerebral cortex. (*Pediatr Res* 20: 391–397, 1986)

Abbreviations

βNGF, β-nerve growth factor
NGF, nerve growth factor
PC-12, Pheocromocytoma cell line 12
RIA, Radioimmunoassay
CNS, Central nervous system
SE, Synaptosomal extract

NGF is a protein involved in the life cycle of neurons of the fetal, neonatal, and adult autonomic nervous system (1, 2). When NGF is introduced *in vivo* or in suitable *in vitro* culture systems, it elicits a variety of morphological and biochemical changes in noradrenergic neurons of sympathetic ganglia (3). NGF also elicits similar biological responses in mediodorsal neurons of sensory ganglia of peripheral nervous system (4). Conversely,

NGF-antiserum disrupts the ultrastructure and impairs several biochemical functions leading to the death of the target neurons (5–7). These results provide unambiguous evidence in support of a survival and maintenance role for NGF in neurons of the peripheral nervous system.

In contrast, the nature of NGF target neurons in the CNS is not clear. The findings that exogenous NGF stimulates regenerative growth of central noradrenergic neurons and that anti-NGF inhibits this NGF-stimulated regenerative growth indicate that central catecholaminergic neurons may acquire NGF responsiveness during repair and regeneration processes (8, 9). Furthermore, recent data indicating the ability of NGF to augment choline acetyltransferase activity in neonatal and adult brain (10–12) and in aggregate brain cell culture systems (13) suggest that cholinergic neurons also may represent NGF-target neurons in the CNS. Retrograde axonal transport of NGF has been described in certain central cholinergic systems supporting the view that NGF may function in adult cholinergic neurons (14, 15). Despite the different βNGF biological responses, there is considerable controversy regarding the location and amounts of NGF present in the CNS (16–19).

Earlier studies including our own (18) utilized RIA systems developed against NGF purified by conventional methods and NGF purified in this way may contain immunogenic contaminants (20). Therefore, in the present study, we reexamined the presence of NGF in mouse brain and cortex using a newly developed NGF-RIA system with a highly sensitive and specific polyclonal antiserum raised against an immunogenically pure βNGF preparation. The same highly purified βNGF was utilized to produce labeled antigen. In addition, we assayed for βNGF in purified cerebral cortical synaptosomes (pinched-off nerve terminals), the organelles reported to be rich in NGF-specific receptors (21, 22). Finally, the presence of βNGF was validated using a PC-12 neurite outgrowth bioassay system. The results indicate the presence of βNGF in subnanogram concentrations in brain cerebral cortex and cortical synaptosomes.

MATERIALS AND METHODS

Animals. Time-dated pregnant Swiss Webster mice were purchased from Simonsen Laboratory (Gilroy, CA). On arrival to the Harbor UCLA vivarium, the animals were housed in individual cages and were given standard mouse food and water *ad libitum.* Pups born within 16 h of each other were pooled and randomly distributed eight pups per mother. The day of birth is recorded as day 0. Each litter was housed with its mother in a metal cage with wood shavings for bedding. Animals were inspected daily to guarantee maternal care, and cages were changed three times weekly. The litter size was maintained at eight per dam until the time of weaning. On day 21, the pups were separated from the mother and maintained thereafter as four weanlings per cage. In studies involving animals older than 21 days of age, only female mice were taken for the experiment.

All animals were sacrificed by CO_2 narcosis which ensured death in less than 1 min. Care was taken during the dissection not to reuse the scissors and forceps used for removal of sub-mandibular glands to carry out the dissections of brain and various brain regions.

Whole brains were removed and placed in a beaker containing 5 ml of 0.32 M ice cold sucrose. Brain tissues were rinsed at least three times with 5 ml of 0.32 M sucrose. During dissection, the whole brain was placed on a piece of glass plate overlying an ice bucket. The cortices were dissected free from adhering corpus callosum with fine forceps and scissor and immediately trans-ferred to a beaker containing 0.32 M ice cold sucrose. Usually 2 to 2.5 g wet weight of cerebral cortex was pooled and three such pools for each age were individually processed for fractionation studies.

Isolation of synaptosomal fraction. For synaptosomal isolation the procedures described by Gray and Whittaker (23) and later by Levitan *et al.* (24) were modified as described below. Briefly, pooled cerebral cortical tissues were placed in 9 volumes of 0.32 M sucrose and cut into pieces with a fine scissor. The minced tissue was homogenized (three up and down strokes) gently by hand, and then by a motor driven homogenizer (400 rev/min, eight up and down strokes). Both hand homogenization and motor driven homogenizations were performed in a glass-Teflon homogenizer with a clearance of 0.25 mm. The homogenates were centrifuged as outlined in Figure 1A. The P2 pellet was resuspended in 0.32 M sucrose (5 ml/g original tissue), layered on a discontinuous sucrose density gradient consisting of equal layers of 1.2 and 0.8 M sucrose (12 ml each) and kept 1 h before use. Samples then were centrifuged at 75,000 × *g* for 120 min in a Beckman ultracentrifuge with a SW 25.1 rotor. The fractions between the 0.8 and 1.2 M sucrose interfaces were removed and
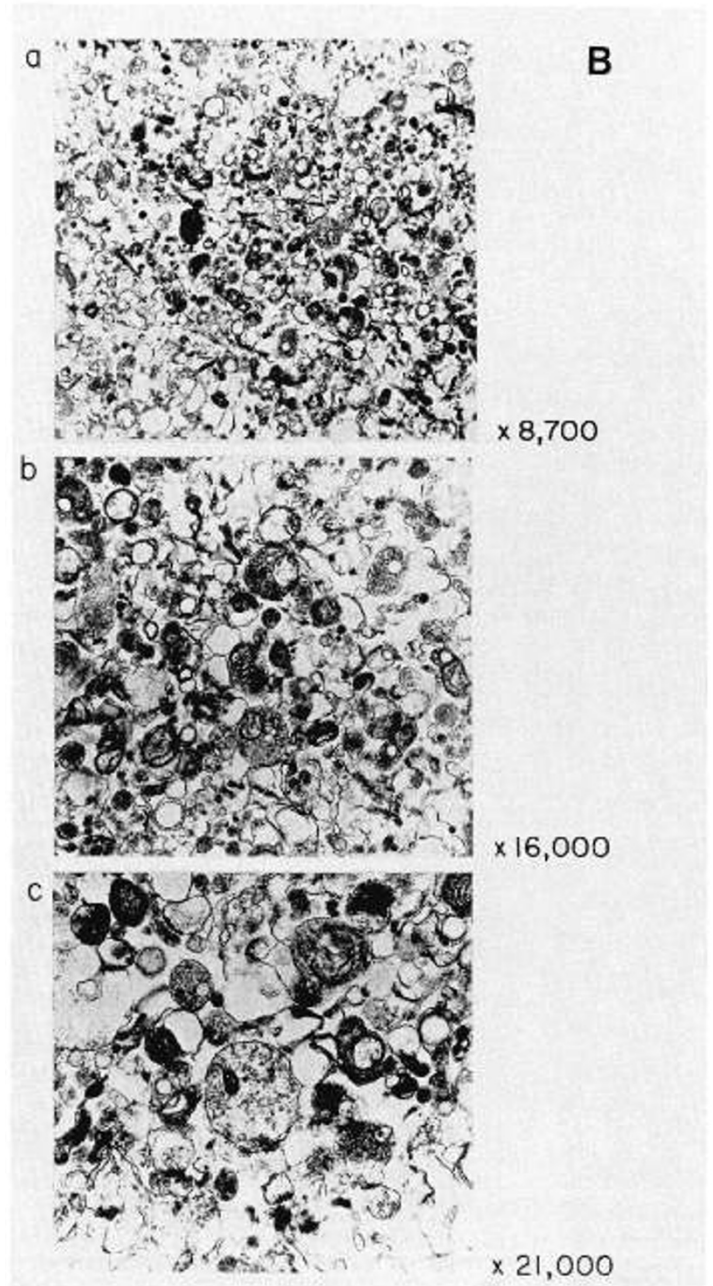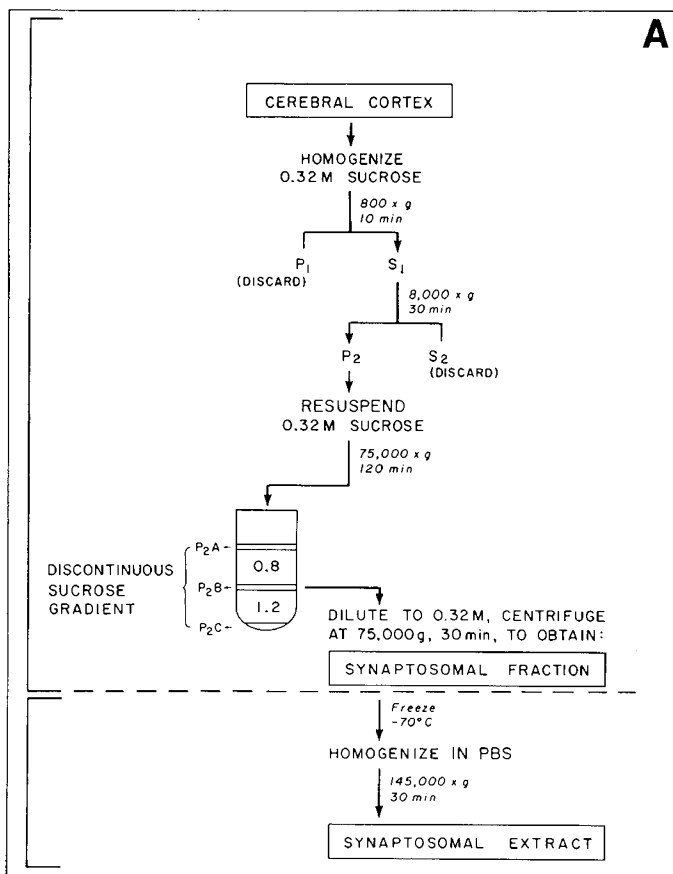

Fig. 1. Isolation of synaptosomes from mouse cerebral cortex. *A*, schematic of the procedure used for isolation of synaptosomes from 26-day-old mouse cerebral cortex. *B*, synaptosomal to actions as observed by electron microscopy. Magnification, *a* = ×8,700, *b* = ×16,000, *c* = ×21,000. See text for other details.

diluted to 0.32 M sucrose by adding 0.16 M ice cold sucrose solution. The synaptosomal fractions were pelleted by centrifugation at 75,000 × g for 30 min. The supernatant solutions were discarded and the pellets resuspended in 0.05 M phosphate buffered saline (1 ml/2 g original tissue) and frozen at −70° C overnight. On the following day, the samples were thawed, transferred to a glass homogenizer, and homogenized (10 up and down strokes). The samples were centrifuged at 145,000 × g for 30 min using a 40.3 rotor. The clear supernatants, referred to as "synaptosomal extract," were used for quantification of βNGF. Protein content was measured by the method of Lowry et al. (25).

Extraction of mitochondrial-rich fractions. For comparative purposes, we also extracted the P2C pellet (mitochondrial-enriched fraction) obtained from the discontinuous sucrose density gradient centrifugation. Homogenization and extraction were performed as described for the synaptosomal fractions.

Isolation of "nonsynaptosomal" fraction. For studies of "nonsynaptosomal fractions," pooled cerebral cortices were subjected to initial homogenization in 0.25 M sucrose, a suboptimal condition for obtaining synaptosomal fractions. The 10% homogenate prepared in 0.25 M sucrose was centrifuged as outlined in Figure 1A for isolation of synaptosomal fractions.

Isolation of synaptosomal fraction after intraventricular injection of βNGF. To assess the extent of possible artifact in the measurement of synaptosomal βNGF concentrations, experiments were performed in animals that received intraventricular injections of purified βNGF preparation. The specific landmarks for intraventricular injection were standardized by injecting 3 μl of India ink and studying the spread of the ink in animals sacrificed at different times. For these experiments, 21-day-old animals of similar body weights were chosen. In experiment 1, mice were injected intraventricularly with 5 ng βNGF dissolved in 3 μl of saline; in experiment 2, 100 ng βNGF were injected in 3 μl of saline. Animals were sacrificed 16 h after the injection. Cerebral cortices were separated and synaptosomal fractions isolated as described in Figure 1A.

Electron microscope technique. The fractions collected at the interface of 0.8 M and 1.2 M sucrose solutions were diluted with 10 volumes of 2.5% glutaraldehyde, pH 7.3, in 0.1 M sodium cacodylate buffer. The mixture was centrifuged at 50,000 × g for 30 min using a SW25.1 rotor. The supernatnat was discarded. The pellets were washed three times with 5 ml of the above buffer, and postfixed in buffered 1% osmium tetroxide for 30 min at 4° C. Pellets were dehydrated and embedded in Epon. Sections stained with toluidine Blue were cut at various levels and examined in a Hitachi H1-11E electron microscope. Photographs were taken from the entire thickness of the pellet.

βNGF RIA. The βNGF antiserum used in the present study was developed in a rabbit by repeated intradermal injections of mouse submandibular gland βNGF prepared according to the method of Mobley et al. (26) as modified by Chapman et al. (27). The same purified βNGF was iodinated by the chloramine T method of Greenwood et al. (28). Labeled βNGF was purified as described in Grueters et al. (29). The specific activity of ^{125}I-βNGF determined by self displacement assay was 5.3 × 10⁶ Ci/mol. The antiserum bound 50% ^{125}I-βNGF at a final dilution of 1:250,000. Binding affinity of the antiserum for βNGF was estimated by Scatchard analysis. There were two populations of antibodies with Kd values of 7.6×10^{11} and 7.0×10^{10} mol/liter. The mean Kd determined by Lineweaver-Burk plot was 1.7×10^{11} mol/liter. The RIA was performed using a liquid phase double antibody method. The buffer and assay protocol were as described by Walker et al. (30), except that 1 h after the addition of goat antirabbit immunoglobulin, 60 μl of 25% polyethylene glycol 8000 (J. T. Baker Chemical Co., Phillipsburg, NJ) was added. One hour later the tubes were centrifuged at 2500 × g for 30 min. The supernatants were aspirated and the precipitate counted in a well type γ spectrometer. The RIA system showed no cross-reaction with ovine growth hormone, ovine prolactin, rat growth hormone, porcine relaxin, oxytocin, vasopressin, glucagon, insulin, submandibular gland renin, angiotensin II, or epidermal growth factor when tested at maximal concentrations of 1 μg/tube. The least detectable concentration of βNGF (defined as the point at which 10% displacement of [125]-βNGF by unlabeled βNGF was achieved) consistently ranged from 16 to 21 pg/tube. Nonspecific binding was consistently less than 6%. Inter- and intraassay coefficients of variation were 5 and 6%, respectively.

PC-12 cell bioassay. PC-12 cells were cultured in medium consisting of 85% RPMI-1640 medium supplemented with 10% horse serum and 5% fetal calf serum, 100 μg/ml streptomycin, and 150 U/ml penicillin. For the neurite bioassay, cells maintained in log phase growth were washed with Ham's saline B and resuspended in RPMI buffer containing 3% horse serum. Aliquots of cells were cultured in the presence of synaptosomal extracts in the absence or presence of purified βNGF antibody. The bioassays were carried out in six well cluster dishes (Costar, Van Nuys, CA). Cells were inspected at the end of 24 h for a neurite outgrowth response.

Statistical analysis. All data were expressed as mean ± SEM. RIA parallelism was assessed by measurement of βNGF in varying amounts of synaptosomal extracts or other fractions to be tested (15–300 μl). The slopes of the regression lines (of percent labeled NGF binding versus NGF standard or volume of test material) were calculated after log-logit transformation and tested for significance using Student's t test. βNGF concentrations between age groups were compared using analysis of variance. Individual comparisons after analysis of variance were made using the Newman Keul's test. Significance was assumed at the 5% level ($p < 0.05$).

RESULTS

The protocol used for isolation of synaptosomal fractions from mouse cerebral cortex is outlined in Figure 1A. The quality of synaptosomal fractions isolated at the 0.8 and 1.2 M sucrose interface was assessed by electron microscope examination. Representative micrographs of these interface fractions isolated from 26-day-old mouse cerebral cortex are shown in Figure 1B. This fraction was found to be rich in synaptosomes, but small amounts of membrane sacs, isolated endoplasmic reticulum, synaptosomal membrane fragments, and mitochondria also were observed. The origin of membrane fragments and free mitochondria is not clear; they may be of nonsynaptosomal origin or may have arisen from rupture of synaptosomes during preparation.

SEs prepared from 21-day-old mouse cerebral cortices were examined for βNGF immunoreactivity. SE, when added in varying amounts, produced RIA displacement curves parallel to the purified βNGF standard (Fig. 2, *upper panel*). The mean slope values for the regression lines generated by standard βNGF and synaptosomal extracts were −1.0 ± 0.01 and −0.96 ± 0.03, respectively ($p > 0.05$). Extracts of the mitochondrial-rich fraction, however, produced a nonparallel line in the βNGF-RIA (Fig. 2, *lower panel*). The mean slope values for the regression lines of standard βNGF and mitochondrial extracts were −1.0 ± 0.01 versus −0.72 ± 0.02, respectively ($p < 0.05$).

The existence of immunoreactive βNGF material in synaptosomal fractions was established by isolated 0.8 and 1.2 M sucrose interface organelles following initial homogenization of cerebral cortices in 0.25 M sucrose, a nonoptimal condition for obtaining synaptosomal fractions. For this purpose, half of the cerebral cortices removed from the same animals were homogenized in 0.32 M sucrose solution. Both of the 10% homogenates were processed as described in Figure 1A. Only SE produced a RIA-displacement line parallel to the βNGF standard (Fig. 3). The slope value for the regression line generated by nonsynaptosomal extract was −0.74 ± 0.01 ($p < 0.05$) relative to βNGF standard.

Because isolation of synaptosomal fractions involved several hours of homogenization and differential centrifugation proce-
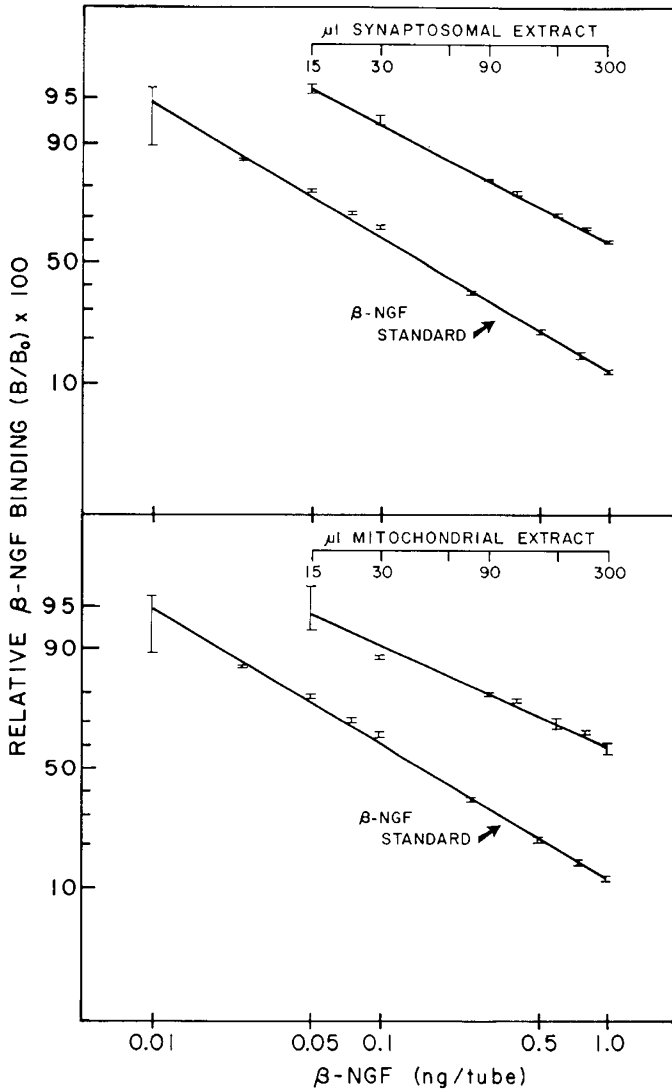
Fig. 2. βNGF immunoreactivity in synaptosomal and mitochondrial fractions of mouse cerebral cortex. Synaptosomal (P_2B) and mitochondrial (P_2C) fractions were isolated from 26-day-old mouse cerebral cortex as described in Figure 1A. Synaptosomal and mitochondrial extracts were prepared in phosphate-buffered saline buffer as detailed in text. Varying amounts of the extracts were assayed for βNGF concentrations. *Note*: the regression lines for displacement by synaptosomal extracts were consistently parallel to the βNGF standard. The regression lines for mitochondrial extract were not parallel statistically. The slope values are given in the text.

dures, certain artifacts, such as exogenous uptake or redistribution among various intracellular compartments cannot be ruled out. To evaluate the extent of artifact, βNGF concentrations were measured in synaptosomal fractions isolated from cerebral cortices of 21-day-old mice which received intraventricular injections of purified βNGF. Exogenous administration of either 5 ng (six times whole brain βNGF content) or 100 ng (120 times whole brain content) βNGF 16 h prior to sacrifice did not appreciably increase synaptosomal βNGF concentrations (Fig. 4).

The relative amounts of βNGF in whole brain, cortex, and synaptosomal extracts of 26-day-old mice are shown in Figure 5. The βNGF concentrations, expressed per milligram synaptosomal protein, were higher in SEs than in whole brain or cortex extracts. βNGF concentrations in synaptosomal extracts of mouse cerebral cortex measured during various stages of development are shown in Figure 6. The mean synaptosomal βNGF concentration was highest on postnatal day 12 and the content
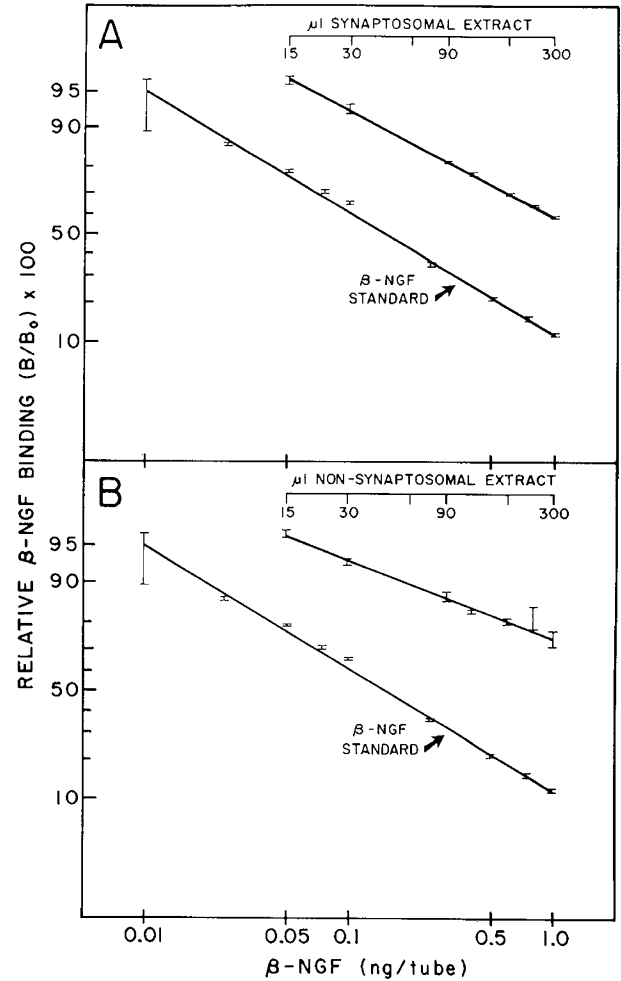

Fig. 3. βNGF immunoreactivity in synaptosomal *versus* nonsynaptosomal fractions. Pooled cerebral cortices of 26 day old mice were divided into two halves. One-half was homogenized (10% w/v) in 0.32 M sucrose and the other in 0.25 M sucrose. Both homogenates were identically processed as in Figure 1A. βNGF content was assayed in varying amounts of the extracts. *A* shows synaptosomal extract and *B* shows nonsynaptosomal extract. Only synaptosomal extract (*A*) produced a displacement line parallel to standard βNGF. Slope values are given in the text.
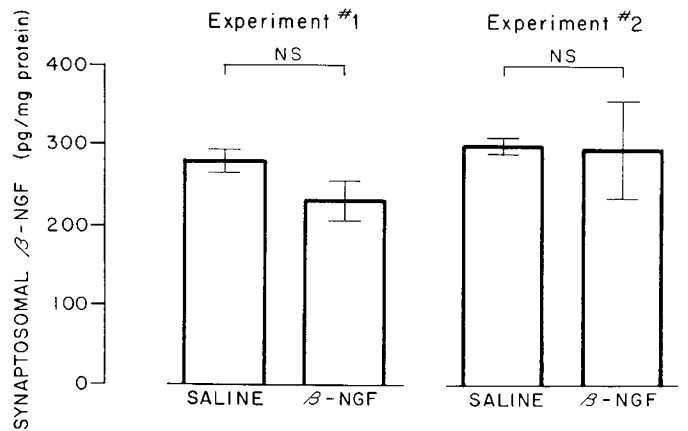

Fig. 4. Synaptosomal βNGF concentrations following intraventricular injection of purified βNGF. Groups of 21-day-old mice (15 animals in each group) were injected intraventricularly with: a) 3 μl volume of sterile saline, b) 5 ng βNGF/mouse in 3 μl saline (experiment 1) or c) 100 ng (βNGF/mouse) in 3 μl saline (experiment 2). Animals were sacrificed 16 h after the injection. Cerebral cortices were removed, pooled (five cortices/pool) and processed for synaptosomal isolation as described in Figure 1A. See text for other details.

decreased with increasing age. No significant differences were seen between days 21 and 32. In contrast to synaptosomal βNGF concentration, the protein content of SEs was unchanged from day 12 to 32.

Neurite outgrowth response to SE. To assess the biological activity of the synaptosomal βNGF, 100 mouse cerebral cortices from 12-day-old animals were processed as described in Figure 1A. Synaptosomal fractions were extracted in RPMI-1640 buffer and concentrated by pressure dialysis against the same buffer using Diaflo ultrafiltration membrane 10YM (Amicon Corporation, Lexington, MA). βNGF concentrations equivalent to 4 g original cerebral cortex (wet weight) were added to wells containing PC-12 cells (1 × 10^4 cells/ml). The neurite outgrowth response was observed after a 24-h incubation period (Fig. 7). The positive neurite outgrowth response due to synaptosomal βNGF was completely inhibited by βNGF-antibody (Fig. 7 D and H). Three independent isolation procedures consistently yielded positive neurite response in the PC-12 cell bioassay system. In all cases neurite outgrowth was accompanied by PC-12 cell aggregation, but βNGF antibody had no influence on the cell-cell aggregation.

DISCUSSION

We chose cerebral cortex for our study since this region has been reported recently to contain NGF-responsive cholinergic neurons both in neonatal and adult animals (10). The protocol described in Figure 1A routinely yielded good synaptosomal preparations as judged by electron microscopic and morphological criteria. We avoided repeated washings and rehomogenization procedures hoping to minimize artifacts.

For synaptosomal βNGF quantification we used a newly developed βNGF polyclonal antiserum consisting of at least two high affinity antibodies. The assay is sensitive to 16–21 pg βNGF/ tube. Synaptosomal extracts produced displacement lines in the RIA parallel to standard βNGF suggesting the presence of βNGF-like immunoreactive material. Both mitochondrial fractions and 0.8 and 1.2 M sucrose interface fractions, following initial homogenization of cerebral cortex in 0.25 M sucrose, produced nonparallel displacement in the βNGF-RIA. The significance of nonparallel displacement needs further investigation. Whether this material represents the high molecular weight form of βNGF (pro-βNGF) or a breakdown product of βNGF remains to be clarified. Moreover, we did not analyze the purity of the non-
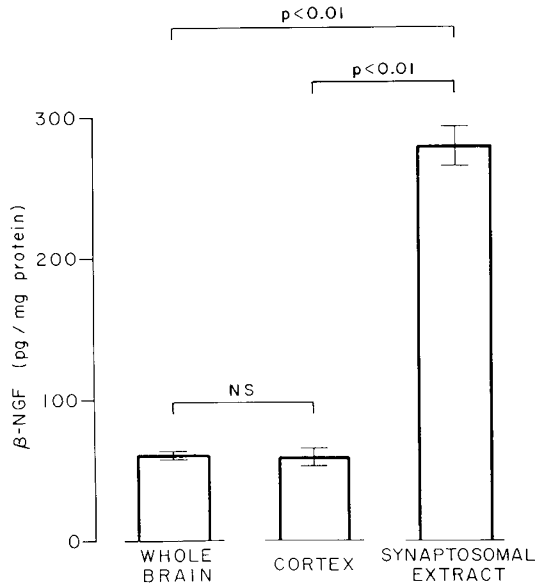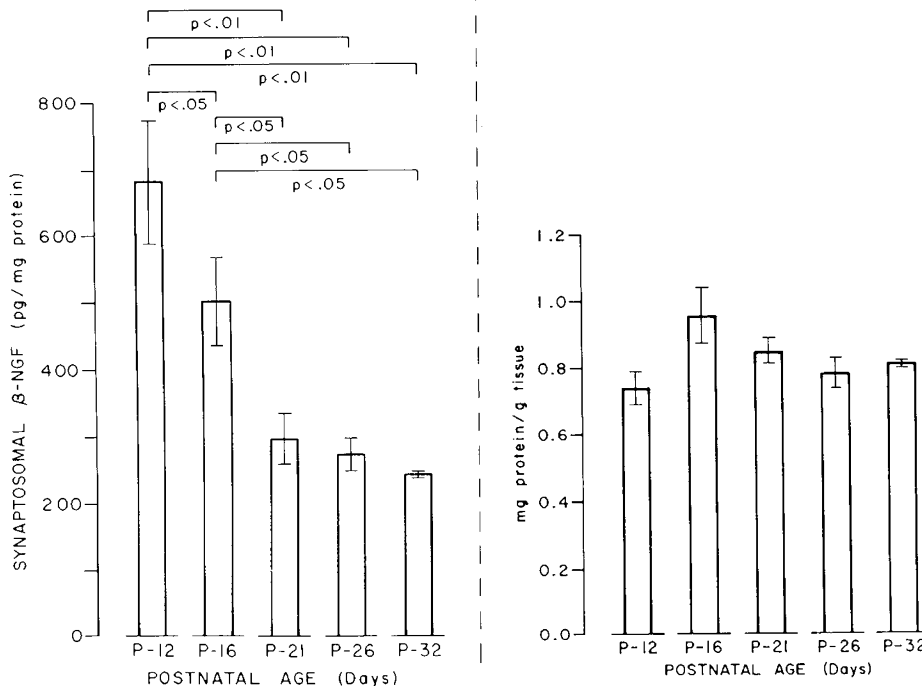

Fig. 5. The relative distribution of βNGF in 26-day-old mouse brain. Extracts of whole brain, cerebral cortex, and synaptosomes of cerebral cortex were prepared in phosphate buffered saline as described in the text. βNGF concentrations were determined by RIA.

Fig. 6. Developmental profiles of synaptosomal βNGF (*left*) and protein concentrations (*right*). Cerebral cortices removed from mice of various postnatal ages (day 12, 16, 21, 26, and 32) were processed for synaptosomal isolation as detailed in Figure 1A. Synaptosomal extracts were assayed for βNGF concentrations by RIA. Protein content in the extracts were measured by the method of Lowry *et al.* (25). See text for other details.
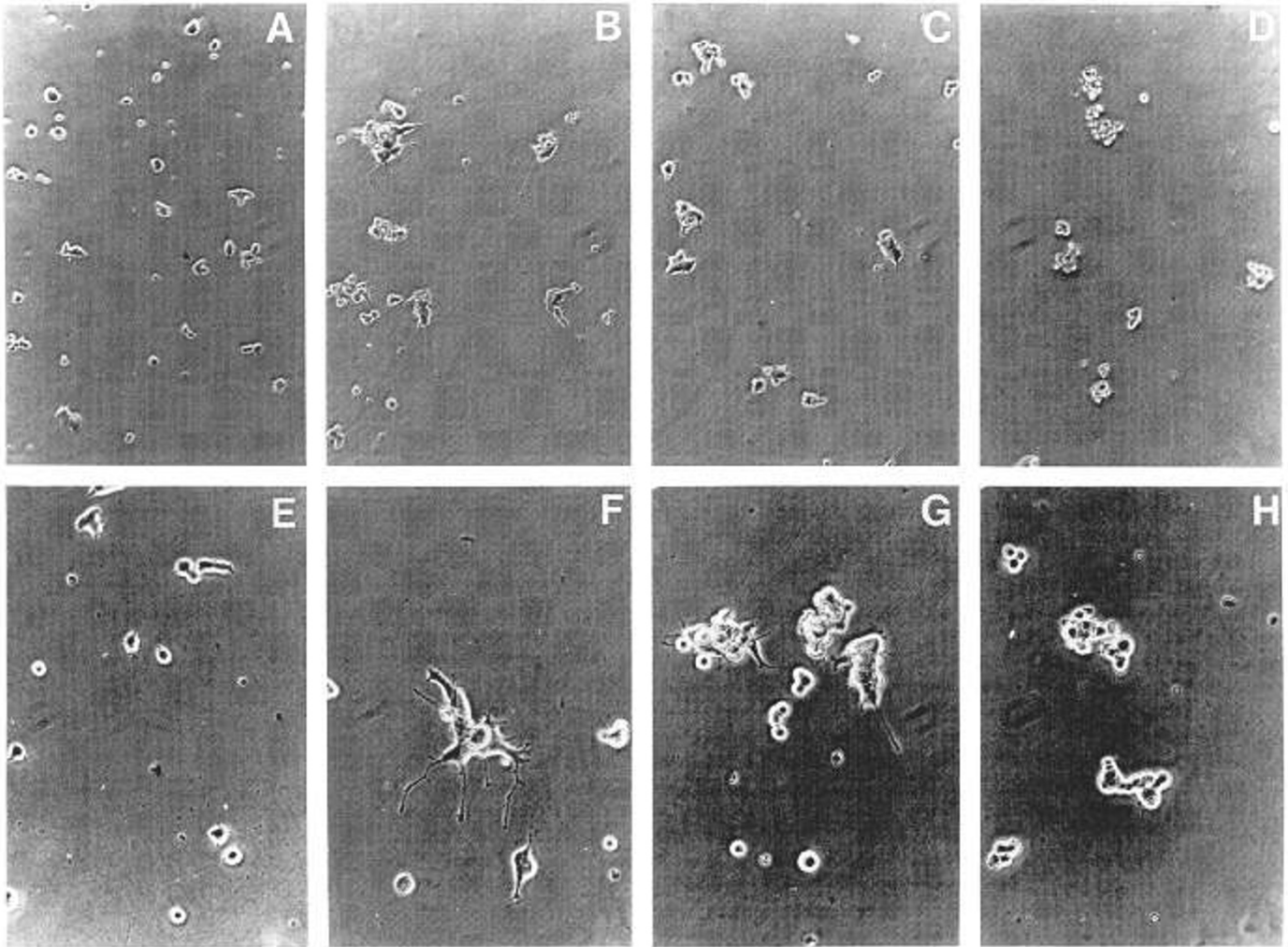
Fig. 7. PC-12 bioassay for SE. Synaptosomal fractions isolated from 12-day-old mouse cerebral cortices were extracted in RPMI-1640 buffer and pressure dialyzed against the same buffer, as indicated in the text. Concentrations equivalent to 4 g original cortex wet weight were added to culture dishes containing 1 ml of PC-12 cell suspension (2×10^4 cells). To assess the specificity of βNGF-induced neurite outgrowth, purified βNGF antibody was included in companion dishes. The neurite response was recorded after 24 h. Cells were photographed using a phase contrast microscope. *Additions:* none (A, E); SEs of two independent preparations (B, C, F, G); SE in the presence of 500 μg of purified βNGF antibody (D, H). *Top row* A–D = $\times 100$ magnification, *bottom row* E–H = $\times 200$ magnification.

synaptosomal fractions. Reports of the presence of NGF-specific receptors in synaptosomal fractions (21, 22), as well as specific retrograde axonal transport in CNS (14, 15), prompted us to examine the extent of any artifact which might be induced by receptor binding in the synaptosomal preparations. Even when large amounts of βNGF (six to 10 times the whole brain βNGF concentration on day 21) were given intraventricularly, we were unable to alter the endogenous synaptosomal βNGF concentrations. This result suggests that βNGF is not freely diffusable from the intraventricular space to other brain regions and that the endogenous synaptosomal βNGF immunoreactivity did not represent contamination occurring during the tissue processing procedures. Preliminary studies revealed that the addition of purified βNGF at a concentration of 5 ng/ml in a 10% cortical homogenate prepared in 0.32 M sucrose did not influence the concentration of endogenous synaptosomal βNGF concentration.

Small, but significant, concentrations of βNGF were detected in whole brain, cortex, and synaptosomal fractions. The βNGF concentrations reported herein for cerebral cortex of 21-day-old mice were significantly lower than the levels reported earlier from our laboratory by Walker *et al.* (31). The difference presumably is attributable to methodological variations. In the present study we used a 145,000 g supernatant for βNGF quantification while Walker *et al.* (31) used a 25,000 g supernatant fraction. In

addition, the purified βNGF used in the present study was prepared by the method of Mobley *et al.* (26), as modified by Chapman *et al.* (27), and was used for immunization, iodination, and as reference standard. Also, we used a newly developed higher affinity antiserum for the RIA system in the present study. Finally, we have taken special precautions to prevent NGF contamination from the submandibular gland, a tissue which contains relatively high concentrations of βNGF even during the weaning stage.

The relatively high concentration of βNGF in synaptosomal fractions, as opposed to whole brain or cerebral cortex, demonstrates that this growth factor is not uniformly distributed in brain tissue. Studies of βNGF concentrations in SEs during various stages of development showed that the concentrations of endogenous synaptosomal βNGF is higher on day 12 than at other ages investigated. We did not attempt to isolate synaptosomes from animals younger than 12 days since there is no morphological evidence for the existence of mature presynaptic nerve terminals in such young animals.

Synaptosomal extracts of 12-day-old mouse cortex consistently elicited neurite responses in the PC-12 bioassay. Moreover, this neurite outgrowth response was completely inhibited by βNGF-antibody. Synaptosomal extracts also caused aggregation of the PC-12 cells, but this aggregation was insensitive to βNGF-anti-

body. Further work is in progress to purify βNGF immunoreactivity from synaptic extracts free of aggregation factor(s).

In conclusion, in the present study we detected subnanogram quantities of βNGF in whole brain, cerebral cortex, and SEs of mouse cerebral cortex using a highly sensitive and specific βNGF-RIA system. βNGF in similar concentrations has been reported recently in different regions of rat brain by Korsching and Thoenen (32). These investigators used a two-site RIA technique. In addition, Shelton and Reichardt (33) have reported detection of βNGF-mRNA in mouse brain. Our results indicate that much of this βNGF material in CNS is localized to presynaptic nerve terminals. Whether βNGF is localized within specific nerve terminals and whether βNGF performs any localized neuromodulator or neurotransmitter-like functions on the presynaptic side of the synapse remain interesting aspects for further study.

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